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Development of a novel oral delivery system of edaravone for enhancing bioavailability

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ABSTRACT

Edaravone (EDR), a strong free radical scavenger, is known for its promising therapeutic potential in oxidative stress (OS) associated diseases, however poor oral bioavailability is the major obstacle in its potential use. Oral liquid dosage form is the most preferred delivery method in paediatric, geriatric and specialised therapies. The present research discusses the development of a Novel Oral Delivery System (NODS) of EDR to enhance oral bioavailability. From preformulation study, solubility, and stability were identified as key challenges and the requirement of an acidic environment and protection against oxidation were found to be critical. The NODS made up of a mixture of Labrasol (LBS) and an acidic aqueous system, was optimized on the basis of solubility and stability study. It can be stored $\leq$40 °C for at least one month. Drug release from NODS was slow, sustained and significantly better as compared to suspension. The significant reduction in metabolism and improvement in permeability across the small intestine were observed with NODS compared to free EDR. The oral pharmacokinetic study showed 571% relative bioavailability with NODS compared to EDR suspension. From the results obtained, NODS is a promising candidate for use in OS associated diseases.

1. Introduction

Oxidative stress (OS) due to an overload of free radicals, is one of the main factors that is involved in the pathogenesis of diseases such as neurological disorders, cardiovascular diseases and cancer (Essick and Sam, 2010; Reuter et al., 2010). A strong free oxygen radical scavenger, like edaravone (EDR) (3-methyl-1-phenyl-2-pyrazolin-5-one, MCI-186,) was shown as a potential therapeutic candidate for diseases associated with OS (Kikuchi et al., 2012, 2013). In 2001, Radicut®, an aqueous based parenteral preparation of having 1.5 mg/mL of EDR developed by Mitsubishi Tanabe Pharma Corporation (Japan), was first approved to treat patients of acute ischemic stroke (AIS) in Japan (Anonymous, 2015a; Lapchak, 2010). Furthermore, orphan designation for undisclosed oral formulation of EDR developed by Netherlands based company Treeway, was granted for amyotrophic lateral sclerosis (ALS) by regulatory agencies including European Medicines Agency (2014), (Anonymous, 2015d) and U.S. Food and Drug Administration (USFDA), (2015), (Anonymous, 2015b). Subsequently, Radicut® has been approved in Japan for ALS treatment from 2015 (Anonymous, 2015a). In addition, its therapeutic potential for Alzheimer's disease has been recently revealed (Jiao et al., 2015).

EDR is being explored extensively in research and has development prospects due to its proven safety and efficacy (Kikuchi et al., 2012, 2013, 2011). Its clinical application is still limited due to lack of oral formulation mainly due to poor oral bioavailability (Rong et al., 2014). Poor solubility, stability and dissolution have been reported as the reasons for this poor oral bioavailability (Zeng et al., 2011). Additionally, it has been categorised as the substrate for P-glycoprotein (Pgp), a transmembrane protein, which could increase its excretion and restrict its distribution and lead to poor permeability (Peff = 3.18 ± 0.0706 10^{-7} cm/s) (Rong et al., 2014). EDR was categorised as a class IV drug as per Biopharmaceutics Classification System (BCS) (Rong et al., 2014). Also, being a substrate of uridine 5′-diphospho-glucuronosyl-transferase (UGT) enzymes, it rapidly undergoes extensive metabolism (Li et al., 2012; Ma et al., 2012). The reported absolute bioavailability of EDR was 5.23% (Rong et al., 2014). To improve its physico-chemical characteristic including solubility, stability, dissolution and permeability, the strategy of complexation with hydroxypropyl-β-cyclodextrin was studied (Zeng et al., 2011; Rong et al., 2014; Sato et al., 2009, 2010). By improving solubility, dissolution and permeability, hydroxypropyl-β-cyclodextrin based formulation

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showed 10.3-fold improvement in oral bioavailability. No information on liquid formulation for oral administration has been reported.

An intravenous infusion is the only commercially available formulation of EDR (Rong et al., 2014). The most preferred in terms of patient compliance is oral route (Shahiwala, 2011). The liquid dosage form has proven to be easily swallowed and therefore of benefit to geriatric and paediatric patients as a delivery method (Liu et al., 2014). For successful liquid formulation, solubility and stability are the two key factors to be considered (Niazi, 2009; Strickley, 2004). The effect of pH on solubility and stability of EDR was not assessed. Similarly, the influence of temperature, light, oxygen, initial concentration of EDR and plasma on the stability of EDR has not been evaluated. Hence, pre-formulation profile needs to be established for the development of Novel Oral Delivery System (NODS) of EDR.

Pharmaceutical excipients can improve the performance of an active compound and its use in pharmaceutical dosage (Fatouros et al., 2007). For NODS of EDR, there is a need for a vehicle which can solubilise the desired quantity, modulate Pgp efflux pump to improve the permeability and inhibit UGT to reduce the metabolism. LBS, a water-miscible surfactant, has been used successfully to improve drug absorption by solubilisation and modulating Pgp efflux pump for drugs such as vancomycin (Rama Prasad et al., 2003a,b), low molecular weight heparin (Rama Prasad et al., 2004), gentamicin (Rama Prasad et al., 2003a,b), and rifampicin (Ma et al., 2011). Moreover, LBS is also proven as a UGT inhibitory excipient (Zhou et al., 2015). LBS has been successfully used to formulate sparingly water-soluble Non-steroidal anti-inflammatory drugs (NSAIDs) piroxicam in Piroflam-Li (Germany). A soft gelatin capsule containing LBS as the solubilising excipient is also reported from Sidmak Laboratories (Strickley, 2004).

The objective of the present research was to develop the NODS of EDR for improvement of oral bioavailability and enhancement of patient compliance. The preformulation profile was established to identify suitable conditions for NODS. The selection of pharmaceutical ingredients and its ratio for NODS was optimised based on solubility and stability study. *In-vitro* performance of NODS was evaluated for protection against extensive metabolism and for permeability enhancement through the small Intestine. The *in-vivo* oral bioavailability of NODS compared to suspension was investigated in adult rats.

2. Materials and methods

2.1. Materials

EDR was purchased from Aladdin Industrial Corporation (Shanghai, China). LBS was gifted by Trapeze Associates Pty Ltd, Gattefosse (Clayton, VIC, Australia). Hydrogen peroxide, octanol, potassium pyrophosphate, Tyrode's solution, ascorbic acid, sodium carboxy methylcellulose, DMSO, conc. HCL, di-sodium hydrogen phosphate, citric acid, perchloric acid, formic acid, uridine-diphosphateglucuronic acid trisodium salt (UDPGA), alamethicin, and D-glucaric acid 1, 4-lactone were purchased from Sigma Aldrich (Sydney, NSW, Australia). Acetic Acid, phosphoric acid, sucrose, magnesium chloride, and sodium hydroxide pellets, were purchased from Chem Supply (Adelaide, SA, Australia) Boric acid was obtained from Optigen Scientific (Adelaide, SA, Australia) and Saline from Pfizer (West Ryde, NSW, Australia). Human Plasma was obtained from BioreclamationIVT (Westbury, New York, USA). Fresh blood samples were collected from 10 to 12 week old male Sprague-Dawley rats and the plasma was extracted. Pierce BCA Protein Assay Kit was purchased from Thermo Fisher Scientific, Australia. Millipore Ultra-Pure Water System was utilised as a source of MilliQ water (Millipore Australia). All the mobile phase components were of HPLC grade. All other chemicals and reagents were of analytical grade.

2.2. Analytical methods

2.2.1. HPLC method

The analytical method from the Japanese Pharmacopeia was used with minor modification (Komiyama, 2012). The samples were analysed using a HPLC system (Shimadzu, Kyoto, Japan) with an Ultraviolet–visible spectrophotometer as a detector (model: SPD-20A), an online degasser (model: DGU-20A3), a system controller (model: CBM-20A), an auto sampler (SIL-20AHT), a pump (LC20AD) and a LC solution Chromopac data processor at the wavelength of 240 nm. A Waters Symmetry^{TM} C18 (3.9*150 mm) (part no WAT046980) analytical column and the mobile phase consisting of methanol, milliQ water and acetic acid in the ratio of 100:100:1 (v/v/v) were used. The flow rate was maintained at the rate of 1 ml/min and sample injection volume was 20 μl. The mean regression equation for EDR for the concentration range of 10–180 $\mu g ml^{-1}$ was $y = 106299x - 355277$ ($r = 0.9999$, $n = 10$), where x and y represent the concentration and the peak area of EDR, respectively.

2.2.2. LC/MS/MS method

The samples were analysed by using a Quadrapole LC/MS/MS (Shimadzu, Kyoto, Japan) system fitted with API 3000 mass spectrometer, LC system having an online degasser (model: DGU-20A3), a system controller (model: CBM-20A), a pump (LC20AD) and an auto sampler (SIL-20AHT); and an Analyst 1.6.2 data processor. The concentration of EDR was estimated by using a reported LC/MS/MS method in literature with minor modification (Ma et al., 2012). The extracts were reconstituted in methanol: water mixture (50:50). The chromatographic separation was carried out by using a phenomenex lura C18 (50 mm × 3 mm × 3 μm) column. The gradient method was used containing mobile phase A (MPA) (5% methanol + 95% water + 0.1% formic acid) and B (MPB) (95% methanol + 5% water + 0.1% formic acid) with the program from 15% MPB initially to 70% MPB in 7.5 min, set to 100% for further 0.5 min, followed by 15% for 2 min. The flow rate and injection volume were 0.2 ml/min and 15 μl respectively with total run time of 10 min. The electro spray ionization (ESI) with negative mode was utilized for mass spectrometry. To gain the desired performance, the optimisation of parameters was carried out with the ionization source. The specific transition of EDR (175.1/133.1) and phenazone (189.1/147.1) (as an internal standard) from precursor ion to ion transition (m/z) were analysed in the multiple reaction monitoring mode. Zero air and nitrogen gas were applied as the source gas and both curtain and collision gas, respectively. The calibration curve was plotted based on the peak areas obtained from the known concentration of the drug and the internal standard. The linearity was observed between the concentrations of 10 to 500 ng/mL.

2.2.3. Determination of EDR glucuronide metabolites (EDR-G)

It has been reported that by the heat-acid treatment, nearly 100% EDR glucuronide is hydrolysed, and EDR was found stable against heat-acid treatment (Mizuno et al., 2007). The hydrolysis using 3N HCL was performed as reported in the literature (Mizuno et al., 2007). The EDR glucuronide concentration was estimated by deducting the EDR concentration from the heat-acid treated EDR (i.e. sum of the EDR and its glucuronide conjugate).

2.2.4. Drug extraction from plasma

To determine the concentration of the EDR in plasma, the plasma (120 μl) was added to 1 ml of McIlvaine buffer of pH 5.4 and extracted by using a dichloromethane-*n*-pentane in the ratio of

3:7 v/v. After separating the organic layer by centrifugation for 3000 RPM for 20 min, it was evaporated under a stream of nitrogen at room temperature (Mizuno et al., 2007). The mixture of water and methanol (50:50) was used to reconstitute the residue.

2.3. Preformulation study

2.3.1. Solubility profile

The aqueous solubility of EDR based on pH was conducted between pH 2–10 by using universal buffer (pH 2–8) and alkaline borate buffer systems (pH 9–10) and compared with milliQ water. The universal buffer solutions were prepared using boric acid, citric acid and phosphoric acid. The pH of final solution was adjusted by using 0.2 M NaOH (Wang et al., 2015). The alkaline borate buffer was prepared as per United States Pharmacopeia guidelines. An excess amount of EDR was added to the glass vials containing 2 ml of vehicle which was covered with aluminium foil and sealed with paraffin film to protect against light and moisture. A mechanical shaker (Model: so4036, Axyos Technologies, Brisbane, Australia) was used for 24 h at room temperature (25 °C). The samples were filtered through 0.45-μm polyvinyl difluoride (PVDF) syringe filters. The filtrate was diluted with mobile phase followed by analysing using UV-HPLC method. All solubility studies were performed in triplicate (Zhang et al., 2014).

2.3.2. Stability study

All stability studies were carried out with 500 μg/mL EDR solution for 21 days in triplicate. The influence of pH on the stability of EDR, was studied between pH 2–10. (Robinson et al., 2015). The EDR solution (2 ml) was prepared in glass vials and covered with aluminium foil and sealed with paraffin film. The samples were stored at 25 °C. Thermal stability was assessed by incubating an aqueous solution to various stability conditions including 4 °C, 25 °C/60% RH, and 40 °C/75% RH (Kuehl et al., 2006). Stability to oxidation was assessed by exposing a solution of EDR in 0.03%, and 3% hydrogen peroxide (H_2O_2) (Blessy et al., 2013). For photo stability, the drug solution was stored in KBF ICH 720 (E2) – constant climate chamber as per ICH recommendation (Group, 1996). The concentration dependent stability was conducted at 50, 100, 500, and 1000 μg/mL (Kuehl et al., 2006). The samples were collected at desired time points, filtered and analysed using UV-HPLC method.

2.3.3. Stability study in human and rat plasma

The 100 μl of EDR solution in water (100 μg/mL) was mixed with the blank rat/human plasma (5 ml) and incubated at 37 °C. The samples were collected at 0, 5, 15, 30, 45, 60, 90, 120, 180, 240, 300, 360, 420, 480, and 1440 min and immediately transferred to ice bath. Later, the extraction was carried out by previously mentioned method in Section 2.2.4. The determination of drug concentration was carried out by LC/MS/MS method (Gualdesi et al., 2014).

2.3.4. Log Po/w determination

The Log Po/w of EDR was determined by using a reported protocol in literature with minor modification (Zhang et al., 2014). The octanol:aqueous solutions (milliQ water, and phosphate-buffered saline (PBS) of pH 7.4 in the ratio of 1:2 (V/V) were prepared. The mixture of the above prepared solutions were stored at room temperature for 24 h for pre-saturation. The known amount of EDR was added to the above solutions and were rotated continuously on a mechanical shaker (Axyos Technologies, Brisbane, Australia) for 120 h at room temperature (25 °C). An aliquot from the aqueous phase was collected and the concentration of EDR was determined by UV-HPLC method. Based on the known distribution of the EDR in the aqueous phase, the concentration of EDR in the octanol phase was determined. A log $P_{o/w}$ value was determined as a logarithmic ratio of EDR in octanol phase to that of the aqueous phase. The ChemBioDraw® Ultra 14 from PerkinElmer was used to determine the calculated $logP_{o/w}$ ($cLog_{o/w}$). All measurements were carried out in triplicate.

2.4. Formulation development

2.4.1. In-vitro *glucuronidation assay*

To evaluate the effect of LBS on the metabolism of EDR, *In-vitro* glucuronidation assay was performed with rat liver microsomes (Pranav Patel and Panchal, 2014; Zhou et al., 2015). The differential centrifugation method was used to prepare rat liver microsomes. After 12 h of fasting of male wistar rat, fresh liver was collected and rinse with cold saline. The homogenisation (Model: Precellys 24 homogenizer, Bertin Technology) of liver was carried out in sucrose solution (0.25 M) (1:4 w/w). After centrifugation (Model: Centrifuge 5415 R, Eppendorf) of homogenates at 9×10^3 relative centrifugal force (RCF) for 0.33 h at 4 °C the supernatant was collected. To separate cytosol and microsomes, ultracentrifugation (Model: Optima™ L-80 XP Ultracentrifuge, Beckman Coulter) was performed at 1.05×10^5 RCF for 1 h at 4 °C. The potassium pyrophosphate buffer (pH 7.4) was used to wash the microsomes followed by ultracentrifugation. The concentration of microsomes was determined by the Thermo Scientific Pierce Bicinchoninic acid (BCA) protein assay kit as per the supplier's instruction. Briefly, 25 μl of standard (20–2000 μg/mL) or unknown samples was pipetted into Thermo Scientific™ Pierce 96-well plate. 200 μl of working reagent (50:1, BCA Reagent A:B) was added to each wells followed by incubating plate at 37 °C for 30 min. The absorbance was measured at 570 nm by using plate reader (VICTOR^3 1420 Multilabel Coulter, PerkinElmer, Massachusetts, USA) (Anonymous, 2015c).

For *in-vitro* glucuronidation assay, the solution containing microsomes (200 μg/mL), 0.95 mg/mL magnesium chloride, 24.94 μg/mL alamethicin, 1.05 mg/mL D-glucaric acid 1,4-lactone, 10.44 μg/mL EDR and its equivalent formulation, was kept in an incubator at 37 °C for 5 min. By adding 2 mM UDPGA, the enzymatic reaction was commenced and continued for 15 min at 37 °C. The cold methanol (400 μl) was mixed with the solution to stop the reaction and to precipitate proteins. Proteins were removed by centrifugation at 1.5×10^5 RCF for 0.16 h at 4 °C. The LC/MS/MS was used to quantify the concentration of EDR and its metabolite EDR glucuronide as mentioned in Sections 2.2.2 and 2.2.3. Borneol was considered as a control at 100 μg/mL.

2.4.2. Study design and sample preparation

The selected aqueous buffer system (0.05 M Citric acid, pH 4) and LBS were mixed volumetrically to form mixtures containing 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100% of LBS. Briefly, an excess amount of EDR was added in 5 ml of vehicle in tightly closed test tube and put in a mechanical shaker for 48 h. The samples were collected at desired time points, filtered and analysed with UV-HPLC. All solubility studies were carried out in triplicate (Yeh et al., 2009).

2.4.3. Physico-chemical characterization of formulation

The selected composition with 3% w/v EDR concentration was assessed against potential for change in pH and precipitation after dilution of 1:10 with MilliQ water (Liang, 2014). The finalised formulation was stored at 4 °C, 25 °C/60% RH, and 40 °C/75% RH. Nitrogen purging was conducted to remove the oxygen from vial before sealed. Samples were collected at various time intervals and filtered through 0.45-mm PVDF syringe filter. The analysis of the samples was performed on UV-HPLC. All studies were carried out in triplicate (Liang, 2014).

2.4.4. In-vitro *release study*

The dialysis bag method was used. EDR solution (30 mg/mL concentration, 1 ml) either in novel liquid formulation or in suspension formulation (0.5% sodium carboxymethyl cellulose) was filled in the dialysis bag (Liu et al., 2011). The dialysis bag with formulation was placed in a glass beaker on magnetic stirrer containing 100 ml of milliQ water at 37 °C and 100 rpm. The samples were collected at scheduled time intervals, filtered and analysed using UV-HPLC. The sink condition was maintained by replacing equal amount of blank buffer after collecting each samples. The study was performed in triplicate.

2.4.5. In-vitro *permeation and metabolism study (Pranav Patel and Panchal, 2014; Zhou et al., 2015)*

Male Sprague Dawley rats (300 ± 25 g) were fasted overnight and sacrificed. The small intestines were removed between 50 mm from the stomach to the ileocecal junction. It was washed with cold Tyrode's solution. The Tyrode solution (1 l) was prepared using 8 g sodium chloride, 0.2 g potassium chloride, 95.20 mg magnesium chloride, 200 mg calcium chloride, 28.4 mg disodium hydrogen phosphate, 1 g sodium bicarbonate and 0.99 g D-glucose. The small part of the intestine was ligated to the glass rod on one end firmly tied with silk and second end kept open to insert the solution. Total 2 ml of Tyrode's solution was added into each sac. After filling the solution, the sac was firmly tied with silk and put in to a beaker having 40 ml Tyrode's solution and a test sample. Borneol was used as a positive control at 200 μg/mL concentration. The whole system was continuously supplied with the mixture of O_2 and CO_2 (95%/5%). The initial concentration of EDR was 50 μg/mL in each test. A 200 μl sample was collected from both sides i.e. serosal side (inside) and mucosal side (beaker) at different time points (5, 10, 20, 30, 60, 90, and 120 min) and were further analysed by previously developed LC–MS/MS method.

2.4.6. Pharmacokinetic study (Rong et al., 2014)

The male Sprague Dawley rats (300 ± 25 g) were procured from the laboratory animal services (University of Adelaide, Adelaide, Australia), and the animal experiment was evaluated and approved by the University of South Australia (Australia). As the rats need to be acclimatised with the available environment, food, and water, we procured them a week ahead. Before performing a surgery, the rats were anaesthetized. On the neck part and the area closer to the jugular vein, a longitudinal cut was made. The catheter was filled with 20 units/mL solution of heparin saline and inserted into the jugular vein with the tip in the right ventricle. The catheter was fixed in place by sewing the muscles around the stopper. Another end of this catheter was led out of skin between two ears by passing underneath the skin of the neck part. Finally, the catheter was filled with 500 units/mL solution of heparin saline and plugged into the free end of the catheter. After surgery, the rats were kept individually (in different cages) in order to attain better recovery. Before administering the drug, the animals were fasted for 12 h and had with free access to water. A required amount of EDR was added into 0.5% sodium carboxymethylcellulose solution to prepare the suspension followed by vortexing for approximately 10 min to get homogeneity. Two groups of rats were orally administrated EDR suspension and NODS at an equivalent dose of 30 mg/kg EDR. At the predetermined time interval of 0, 5, 15, 30, 45, 60, 90, 12, 180, 240, 300, 360, 420, and 480 min, a 0.2 ml blood sample was collected and flushing of the catheter was done with an equal amount of the heparin saline solution. The plasma was extracted from the blood samples by centrifugation at 5000 rpm for 15 min at 4 °C and stored at −80 °C until analysis. The analysis for EDR was performed by using LC–MS/MS as mentioned above.

The various pharmacokinetic parameters including maximum plasma concentration (C_{max}), time to achieve maximum plasma concentration (T_{max}), elimination half-life ($t_{1/2}$), area under the concentration–time curve (AUC), and relative bioavailability were determined. Relative bioavailability is defined as a measurement of the bioavailability of EDR suspension B (estimated as AUC) in comparison with NODS of EDR following an oral administration. F_{rel} (%) is calculated by the formula $[(AUC_A \times Dose_B)/(Dose_A \times AUC_B)]$ (John et al., 2013).

2.5. Statistical analysis

Statistical analysis was performed using GraphPad Prism 6. All values were indicated as Mean ± standard deviation (SD). The statistical analysis of data was performed by using Student's *t*-test for two groups, and one-way and two way analysis of variance (ANOVA) for multiple groups. A p value < 0.05 was considered as a significant difference for data analysis.

3. Results and discussion

In the last three decades, researchers over the world have recognised the extraordinary therapeutic potential of EDR for its free radical scavenger activity (Kikuchi et al., 2012, 2011; Tabrizchi, 2000). The limited attention towards defining its physicochemical characteristics and unfavourable oral pharmacokinetic profile creates major obstacles to assess its therapeutic value for various OS associated diseases at the clinical stage. The pre-formulation study to determine the unrevealed physicochemical properties was considered as an initial step for the drug development of EDR.

3.1. Preformulation study

3.1.1. Solubility profile

The water solubility of EDR was found 1.85 ± 0.15 mg/mL. The solubility in aqueous buffers were 1.58–5.80 mg/mL and depended on the pH of the solution pH > 7 at 25 °C (Fig. 1). A significant enhancement of solubility was observed from pH 8 to 10 compared to water solubility ($P < 0.001$) while from pH 2 to 7, there was no considerable difference observed. We have used solubility in water as a control. The phenol like structure of EDR and weak acidic nature having pKa value of 7 allows its ionisation in the basic environment (Higashi et al., 2006; Yoshida et al., 2006). The solubility of the drug could be dependent on the ability to dissociate into ions when subjected to the pH of the aqueous solution (Beringer, 2006). This could be one of the reasons for high solubility above pH 8 because increment in the solubility is dependent on the higher degree of ionization compared to neutral and acidic pH (Zhang et al., 2014). Observations in neutral and acidic environments showed low solubility compared to a basic

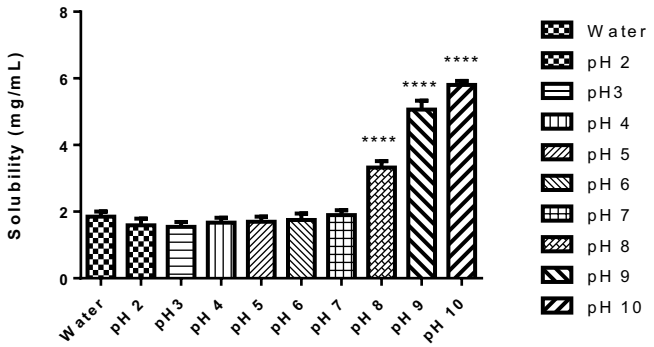

Fig. 1. pH-solubility profile of EDR at various pH in different buffer solutions (mean ± S.D., n = 3). ****$P < 0.0001$ compared to control (Water). One-way ANOVA and Sidak's multiple comparisons test.

environment. So, use of a basic environment could be an option to increase the solubility of EDR in an aqueous solution.

3.1.2. Stability study

To define the stability profile of the drug molecule is considered a mandatory requirement as per the Food and Drug Administration (FDA) and International Conference on Harmonisation (ICH) guidelines (ICH Q1AR2) for registration as a drug. It can influence its safety and efficacy. As per the guideline, the stability study should be conducted for a duration of 6 months (intermediate and accelerated) and 12 months (long term) respectively (Group, 2003). The forced degradation study could be done within a short period of time (a few weeks) and gives an indication of stability in various environmental conditions similar to intermediate, accelerated and long term studies. The determination of a stability profile could help to decide the suitable conditions for the pharmaceutical dosage form (Blessy et al., 2013). Since we would like to develop an oral liquid formulation of EDR, we have used conditions like different pH, temperatures, presence of light and oxygen and effect of initial concentration.

The process of the breakdown of a compound in presence of water is called Hydrolysis. It is considered as one of the most common reactions involved in the degradation process. The range of pH from acidic to basic using different buffer conditions could trigger the process of hydrolysis (Blessy et al., 2013). We have used a universal buffer system (pH 2–8) containing citric acid, boric acid and phosphoric acid (Wang et al., 2015); and alkaline borate buffer system (pH 9–10) (Robinson et al., 2015) to cover the broad range of pH 2–10.

The result of the effect of hydrolysis on EDR was presented in Fig. 2A. EDR was found to be relatively stable from pH 2 to 4 for 21 days as the degradation was <3% which is not statistically significant compared to day 0 sample preparation. At pH 5, there was no significant degradation observed up to 14 days at room temperature and nearly 4% ($p < 0.05$) until 21 days. The significant degradation was seen after day 7 at pH 6 and 7 ($p < 0.01$ on day 14 and $p < 0.0001$ on day 21), after day 3 at pH 8 ($p < 0.05$ on day 7, $p < 0.0001$ on day 14 and 21), after day 1 at pH 9 ($p < 0.05$ on day 3, $p < 0.001$ on day 7, $p < 0.0001$ on day 14 and 21), and 10 ($p < 0.001$ on day 3, $p < 0.0001$ on day 7, 14 and 21) respectively. Two-way ANOVA analysis by using Sidak's multiple comparisons test was performed. In acidic conditions (pH 2–6), EDR was found to be relatively stable with <8% degradation within 21 days compared to <10% in neutral conditions (pH 7) and ≤20% in basic conditions (pH 8–10) (Robinson et al., 2015). EDR seems to degrade rapidly due to basic hydrolysis which was already reported with drug having phenolic structure (Beringer, 2006; Friedman and Jurgens, 2000). It can be ionised in basic conditions and undergoes rapid degradation. Acidic conditions could be preferable for NODS.

The stability study was conducted on EDR with conditions like 4 °C, 25 °C/60%RH, room temperature, 40 °C/75% RH in order to assess the effect of thermal stress conditions as recommended by ICH guideline. The effect of temperature on EDR was not significant as shown in Fig. 2B as <3% degradation was observed at all conditions. The photo stability of EDR was also conducted by exposing EDR to light. Photo oxidation of the drug could happen in presence of light via free radical mechanism (Blessy et al., 2013). EDR showed <3% degradation up to day 21 which was not statistically significant compared to day 0. Two-way ANOVA analysis by using Tukey's multiple comparisons test was conducted.

The oxidative degradation of the drug is also considered as a major concern due to electron transfer leads in the formation of reactive anions and cations (Hovorka and Schoneich, 2001). To define the stability profile of EDR in presence of oxygen, hydrogen peroxide (H_2O_2) was used as an oxidizing agent at 0.03% and 3% concentration at room temperature (Zhang et al., 2014). With 0.03% concentration of H_2O_2, the significant degradation was observed after day 3 with more than 3% degradation ($p < 0.05$ on day 7, $p < 0.01$ on day 14 and $p < 0.0001$ on day 21) to nearly 12% degradation until day 21 as showed in Fig. 2C. In presence of 3% H_2O_2, EDR suffered significant degradation with >7% on day 1 to

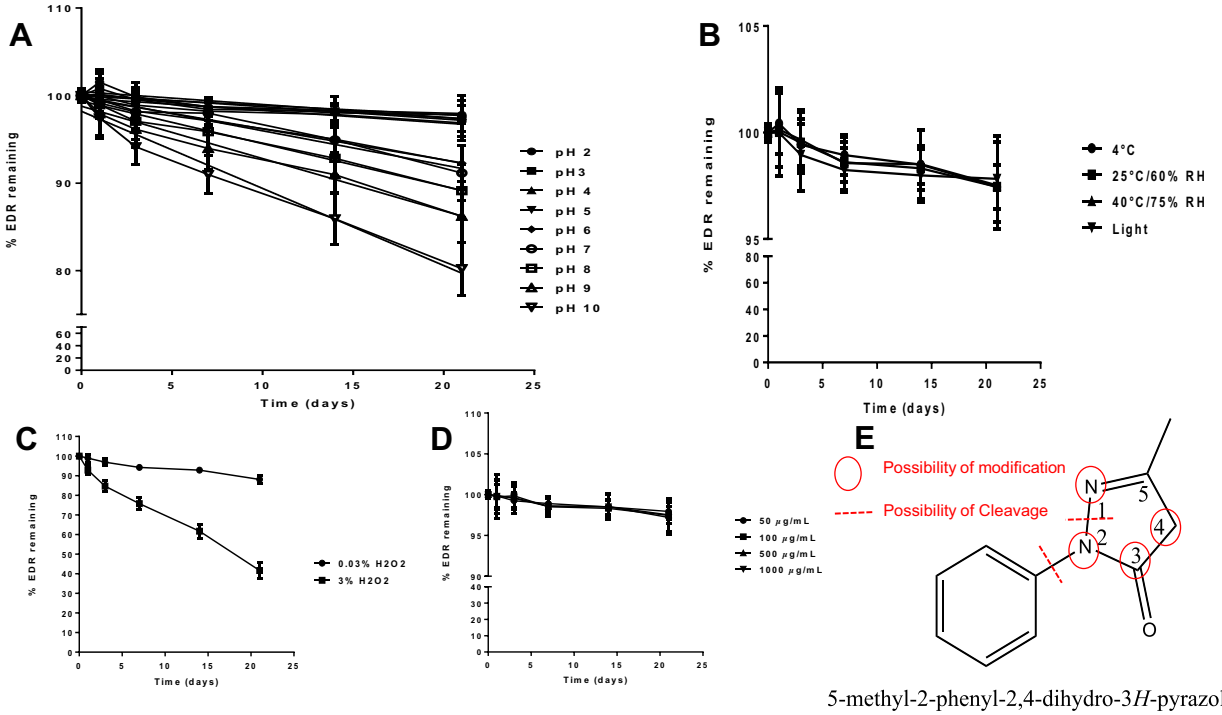

Fig. 2. Stability profile of EDR at different pH dependent stability study (A), Thermal and Light stability study (B), Oxidative stability study (C), Concentration dependent stability study (D), chemical structure of EDR with possible modifications due to Hydrolytic degradation and oxidative degradation (E) (mean ± S.D., n = 3).

nearly 60% till day 21 ($p < 0.01$ on day 1, $p < 0.0001$ on day 3, 7, 14, and 21). Two-way ANOVA analysis by using Sidak's multiple comparisons test was performed. As EDR has a phenol like structure, it is susceptible to oxidation via electron transfer (Harjivan et al., 2014). Hence, it can be inferred that protection against oxidation is critical for the development of NODS.

The drug like Imexon showed concentration dependent stability as it acts as a catalyst for its own degradation (Kuehl et al., 2006). To evaluate the effect of initial concentration of EDR, different concentrations from 50 to 1000 μg/mL were prepared. The data represented in Fig. 2D, revealed the negligible effect of initial concentration in solution on stability of EDR. Less than 3% degradation was observed until day 21 in all cases, which was not statistically significant compared to day 0. Two-way ANOVA analysis by using Sidak's multiple comparisons test was performed.

EDR is susceptible to alkaline hydrolysis degradation and oxidative degradation. The modification of EDR chemical structure (Fig. 2E) could be occurred due to a reaction with water (Hydrolysis) and presence of an oxygen with electron transfer mechanism (Oxidative) (Hovorka and Schoneich, 2001; Blessy et al., 2013). The cleavage of chemical bonds at position 1 and 2 of pyrazole ring might be resulted into ring opening due to attack of nucleophile. The modification at position 3 and 4 could explain from the proposed mechanism of EDR's free radical scavenger activity (Watanabe et al., 2008).

Poor oral bioavailability of EDR with rapid clearance was reported but the clear mechanism behind it still remains unexplored (Li et al., 2012; Rong et al., 2014). In our experiment, nearly 30% and 50% degradation of EDR in 24 h were observed in human and rat plasma, respectively (Fig. 3). A significant difference in degradation of EDR in human plasma was observed compared to rat plasma after 30 min. Two-way ANOVA analysis by using Sidak's multiple comparisons test was performed. Based on the data, half-life of several hours could be expected. However, half-life of 1.41 h was reported (Rong et al., 2014). Results suggest that other mechanisms like rapid metabolism in liver, and substrate for Pgp might have a major contribution compared to instability in plasma for quick clearance from the body

3.1.3. Log $P_{o/w}$ determination

The absorption of drug depends on its permeability across the biological membrane after dissolution. The prediction of drug permeability is generally determined based on Log Po/w study at the initial stage of drug development. Octanol has lipophilicity similar to that of biological membranes. Log Po/w value of drug determines its ability to partition in to the biological membranes (Fan et al., 2015; Zhang et al., 2014). The partition coefficient value of EDR in water, and PBS (pH 7.4) were 1.20, and 1.10, respectively. The value is comparable to log P (1.16) determined by Chembiodraw ultra 13.0 (developed by PerkinElmer Inc.). The lipophilic nature of EDR was confirmed which could facilitate permeability. In contrast, Rong et al. reported that EDR had poor permeability (Peff = 3.18 ± 0.0706 10 − 7 cm/s). EDR belongs to a class of Pgp substrate which could be the potential reason for its poor permeability (Rong et al., 2014).

3.2. Formulation development

The encouraging therapeutic potential of EDR against various OS associated disorders such as AIS, ALS and Alzheimer disease, will surely lead to a rise in the market share of EDR at a global scale. From our study and literature review, poor aqueous solubility and stability, rapid and extensive metabolism, and poor permeability could be considered as the major obstacles for an effective EDR therapy. The requirement of an acidic environment and protection against oxidation should also be considered as critical factors for the development of oral formulation of EDR.

The NODS based on co-solvent strategy is considered as the most preferable one due to its inherent advantages like low cost, ease of self-administration, easy of manufacture and scale up and better safety profile (Panigrahi, 2012). The acidic aqueous preparation with appropriate co-solvent was considered as an ideal choice for EDR. From literature review, LBS has been selected for study as a co-solvent for EDR as it could improve the oral bioavailability directly or indirectly facilitating solubilisation, protection against rapid and extensive metabolism as well as improving permeability. LBS has a carbon chain length of C8–C10 and Hydrophilic-Lipophilic Balance (HLB) value of 14. It is obtained by the polyglycolysis of medium-chain triglycerides containing 30% fatty acids, 50% esters of polyethylene glycol and 20% free PEG 400. The coconut oil like medium chain triglycerides, caprylic/capric acid and PEG 400; and alcoholysis/esterification process are used to synthesize LBS (Koga et al., 2006; Ma et al., 2011; Rama Prasad et al., 2004, 2003a,b). LBS has a proven its safety via oral

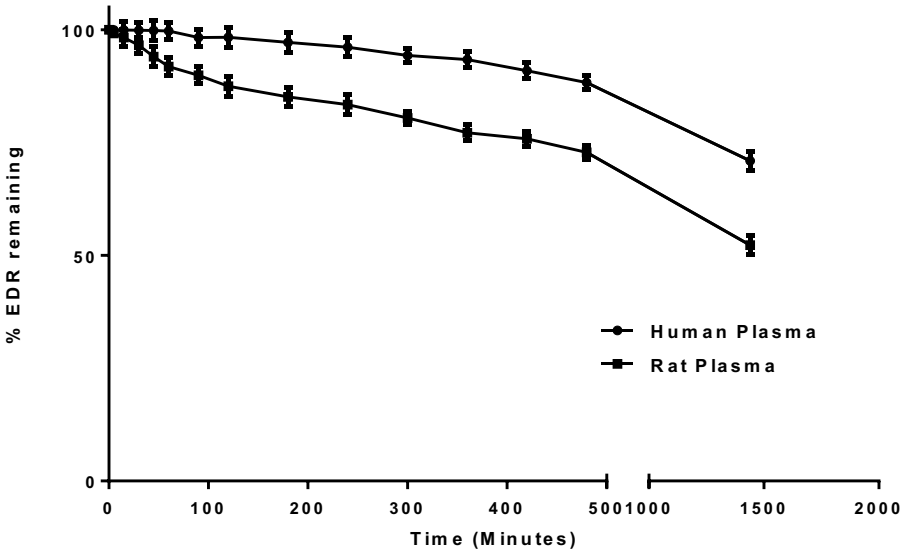

Fig. 3. Human and Rat plasma stability study of EDR (mean ± S.D., n = 3).

administration with 22 g/kg of LD_{50} value in rats and a dose of 1 g/kg/day in dogs for 13 weeks (Shah et al., 2014).

3.2.1. Inhibition of EDR glucuronidation by LBS

LBS as a UGT inhibitory excipient, has showed inhibitory effect on resveratrol glucuronidation (Zhou et al., 2015). The extensive metabolism of EDR via glucuronidation was reported in the literature (Ma et al., 2012). The effect on metabolism of EDR was assessed in the presence of LBS compared with borneol as a positive control on glucuronidation in rat liver microsomes. There was a significant inhibition on glucuronidation observed in the presence of LBS (88.78%) compared to borneol (37.56%) as shown in Fig. 4. The chemical structure of EDR and its glucuronide metabolite are mentioned in Fig. S1. Considering the significant inhibitory effect of LBS on EDR's metabolism, the NODS based on LBS as a co-solvent was selected for further development.

3.2.2. Selection of the composition

The enhancement of solubility of EDR was studied followed by performing a stability study to optimise the ratio of acidic aqueous solution and LBS. Table 1 shows the enhancement of solubility in terms of enhancement factor of EDR in a co-solvent system based on the increment in proportion of LBS. The solubility of EDR in LBS was 62.48 mg/mL. The probable reasons of the enhancement of solubility by LBS could be direct co-solvent effect and micelle formation (Ito et al., 2005; Panigrahi, 2012; Strickley, 2004).

3.2.3. Physico-chemical characterization of NODS

The stability of liquid formulation is always considered a major concern for the development as a market formulation (Niazi, 2009). It was assessed for 1 month as per ICH guideline and evaluated by comparing the drug content, pH and appearance from day 0 (Garg et al., 2007; Liang, 2014). As shown in Fig. 5, no statistically significant difference was observed in EDR content at various storage conditions for 1 month. There was no significant difference in pH of the NODS or precipitation observed until 1 month. Two-way ANOVA analysis by using Sidak's multiple comparisons test was performed.

3.2.4. In-vitro *release study*

The release study was carried out to evaluate the effect of formulation ingredients of NODS on release of EDR compared to suspension. The release of EDR from NODS and suspension are shown in Fig. 6 as a function of time. The burst release was observed in the case of suspension as about 80% cumulative amount compared to 30% within 60 min. The sustained release was observed from NODS suggesting a high affinity between EDR and LBS. LBS has a polyethylene glycol chain which could restrict the release of the drug after micellization (Zhou et al., 2015). At 480 min, EDR was released nearly 90% from NODS compared to 40% from EDR suspension. The significant difference was also observed at all time points except 60 and 90 min ($p < 0.001$). Two-way ANOVA analysis by using Sidak's multiple comparisons test was performed.

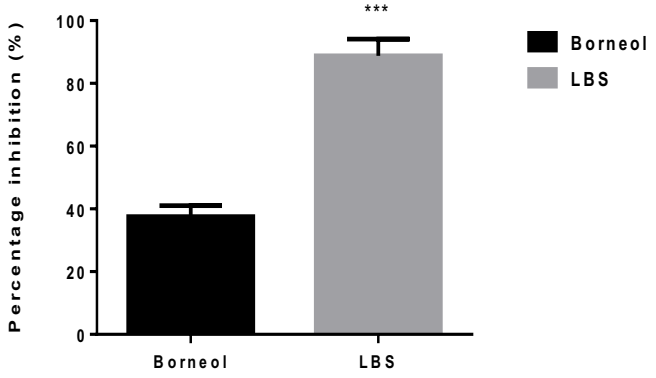

Fig. 4. Inhibitory effect of LBS (1%) on EDR glucuronidation in a microsomal incubation assay (mean ± S.D., n = 3). ***P < 0.001compared to control (Borneol). Unpaired *t*-test (two-tailed).

3.2.5. In-vitro *permeation and metabolism of EDA across the rat small intestine*

The effect of formulation ingredients on EDR permeability and metabolism during permeation across the small intestine was assessed. NODS showed 5 and 2.5 fold higher amount of EDR transferred within 2 h compared to free EDA and with Borneol, respectively (Fig. 7A). NODS and Borneol showed nearly 50% and 74% amount of EDR-G on serosal side compared to free EDR (Fig. 7B). The extensive glucuronidation of EDR was observed during the permeation of EDR. The molar ratio of EDR-G to EDR was 28% and 56% with NODS and Borneol, respectively compared to free EDR (Fig. 7C). The presence of LBS could be the reason for the desired outcome of NODS due to its significant inhibitory effect on glucuronidation. The transfer rate of EDR was 3.73 and 1.41 fold higher with NODS compared to free EDR and Borneol (Fig. 7D). LBS showed an inhibitory effect on Pgp efflux (Lin et al., 2007). It showed the significant role of Pgp efflux inhibitor in improvement of bioavailability of EDR (Rong et al., 2014). An increase in the permeability could be due to the synergistic effect on glucuronidation as well as Pgp efflux effect of LBS.

3.2.6. Pharmacokinetic study

The plasma profile of EDR with time for EDR suspension and NODS are shown in Fig. 8. Various pharmacokinetic parameters were obtained from Phoenix WinNonlin software are presented in Table 2. A significant difference was observed in the pharmacokinetic parameters including C_{max}, $t_{1/2}$, $AUC_{0\text{-}t}$, $AUC_{0\text{-}\infty}$, F0-t (%) and F0-∞ (%). The statistical analysis was performed with two-tailed unpaired *t*-test. The C_{max} value of NODS was 4.41 fold higher than the EDR suspension but no difference in t_{max} value for both formulations was noted. The $t_{1/2}$ value was increased by 1.30 fold in case of NODS compared to EDR suspension. The long $t_{1/2}$ value might be explained by the fact that LBS has an ability to form micelles and EDR could be entrapped into the micelles. The sustained release from NODS was also observed in *in-vitro* drug release study which could be responsible for long plasma half-life of NODS *in-vivo*. The enhancement of oral bioavailability of NODS was 5.75 fold compared to EDR suspension.

A characteristic melting point peak at 128.39 °C in Differential Scanning Calorimetry (DSC) analysis (Fig. S2), sharp numerous distinct peaks in X-ray powder diffraction (XRD) analysis (Fig. S3) and irregular shaped crystals in Scanning Electron Microscopy (SEM) analysis (Fig. S4) study confirmed the crystalline nature of EDR. Due to crystalline nature, the solubility of EDR in suspension formulation was 1.89 mg/mL and additional EDR would be present in solid undissolved form. In case of NODS, EDR was completely solubilised which could be potential reason for enhancing oral bioavailability. The reduction in metabolism and improved permeability in G.I tract might serve to justify the favourable outcome. In conclusion, NODS looks to be a promising candidate for clinical development in the near future.

4. Conclusions

To enable the clinical assessment for various OS associated diseases, the suitable oral liquid formulation of EDR may be considered as a very important. From the preformulation study and based on the available resources; poor solubility, stability,

Table 1
Solubility profile of EDR in Aqueous buffer-LBS mixture (mean ± S.D., n = 3).

Aqueous buffer (%V/V)	LBS (%V/V)	Solubility of EDR (mg/mL) (Mean ± S.D., n = 3)	Enhancement factor[a]
100	0	1.65 ± 0.19	1
90	10	7.18 ± 1.05	4.35
80	20	15.21 ± 1.52	9.22
70	30	21.24 ± 2.85	12.87
60	40	27.28 ± 2.56	16.54
50	50	33.31 ± 1.89	20.19
40	60	39.34 ± 2.74	23.85
30	70	44.38 ± 2.48	26.90
20	80	50.41 ± 1.56	30.56
10	90	55.54 ± 1.11	33.67
0	100	62.48 ± 1.84	37.87

[a] Enhancement factor: the ratio is calculated by EDR solubility in 1 ml of water and LBS mixture/EDR solubility in 1 ml of water.

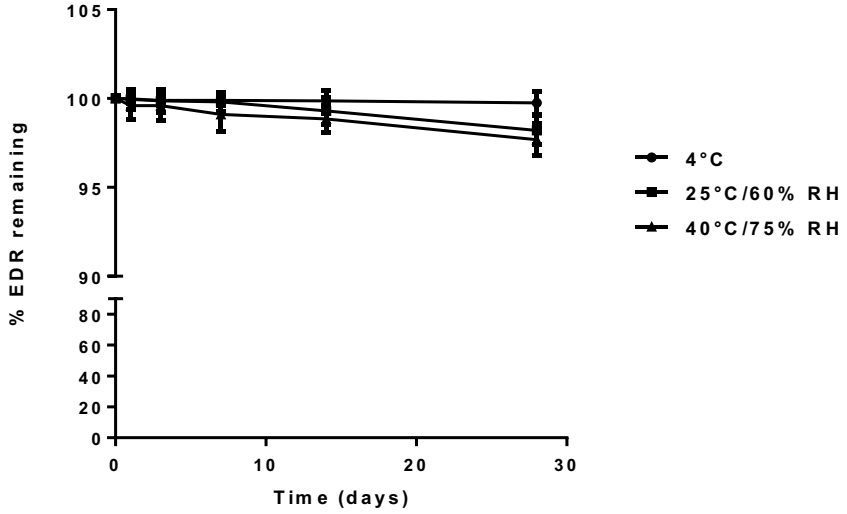

Fig. 5. Stability study of NODS as per ICH guideline (mean ± S.D., n = 3).

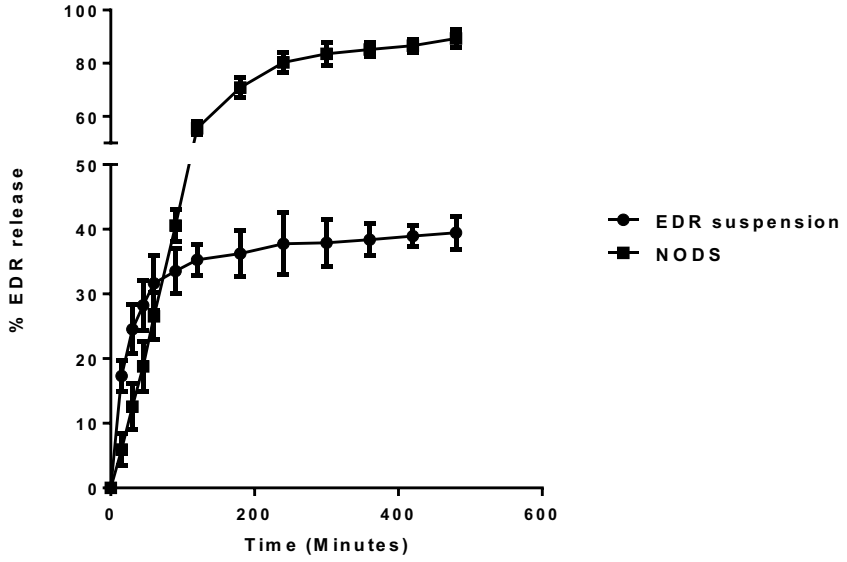

Fig. 6. In-vitro release of EDR from EDR suspension or NODS (mean ± S.D., n = 3).

permeability across the GI tract and extensive metabolism; were determined as the potential obstacles that lead to the poor oral bioavailability. The NODS based on LBS and aqueous buffer system having pH 4 was developed based on solubility and stability study. From the *in-vitro* study, LBS showed a significant improvement in the metabolism of EDR as well as intestinal absorption. The beneficial effect of LBS might be due to its inhibitory activity on UGT enzyme and Pgp efflux transporter. The 5.7 fold improvement

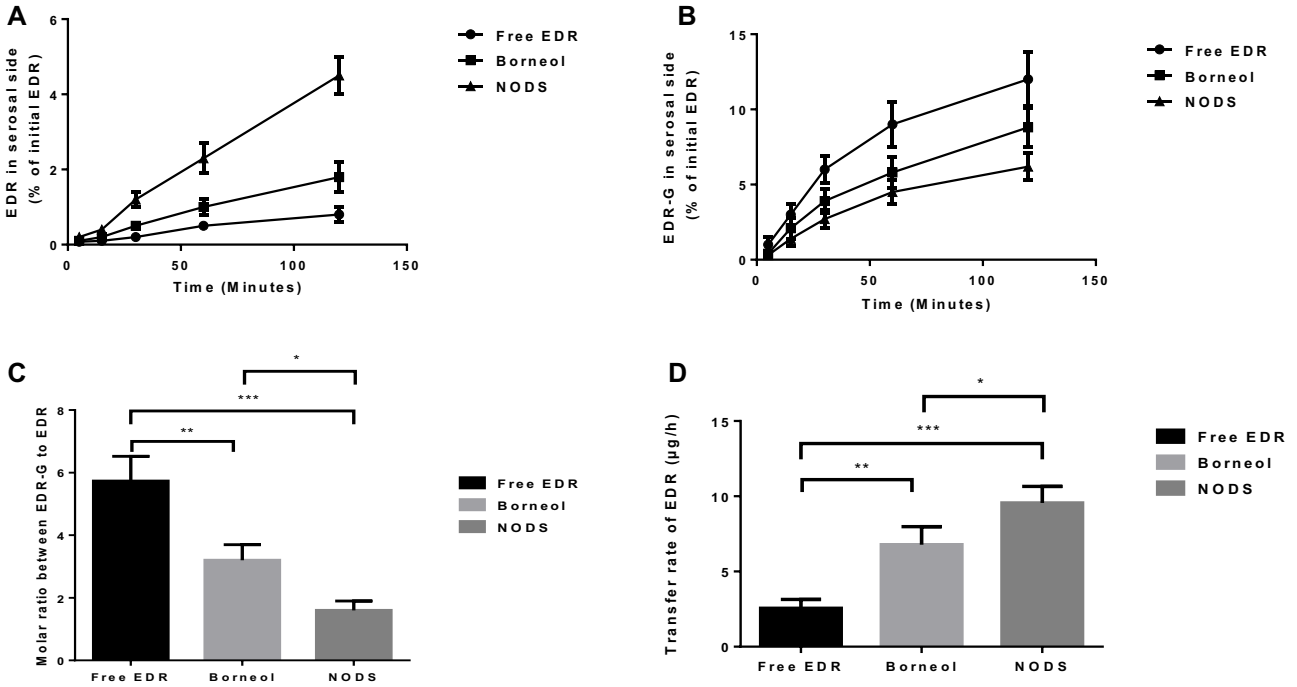

Fig. 7. The effect of formulation ingredients on permeability and metabolism of EDR during transportation across everted sacs of rat small intestine. The amount in percentage of EDR (A) and (B) on serosal side of everted sac at various time interval, molar ratio between EDR-G and EDR (C), and transfer rate of EDR in the serosal side of rat everted gut sacs (D) (mean ± S.D., n = 3). *P < 0.05, **P < 0.01 and ***P < 0.001. One-way ANOVA and Sidak's multiple comparisons test.

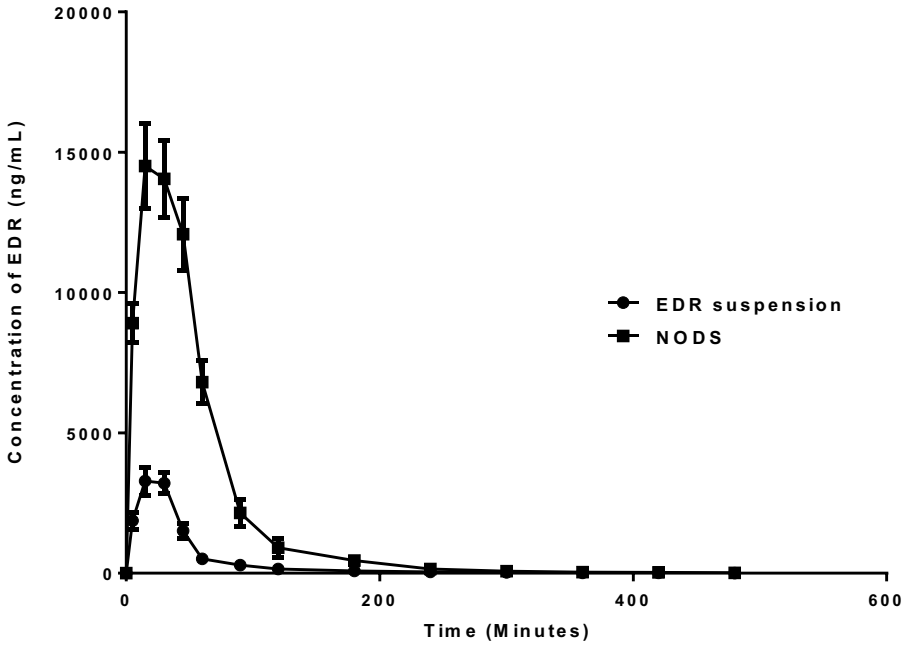

Fig. 8. Plasma concentration-time curves for the EDR suspension and NODS (30 mg/kg) in SD rats after oral administrations (mean ± S.D., n = 6).

in the oral bioavailability might be due to a combination of improvement in solubility, stability, intestinal permeability and reduction of extensive metabolism. This study reveals the importance of selecting appropriate formulation ingredients which provides an ultimate solution to overcome the obstacles for the clinical development of potential therapeutic agent like EDR.

Conflict of interest

Ankit Parikh, Xin-Fu Zhou and Sanjay Garg are named inventors on Chinese patent 201610149832.9. Fujian Kangshimei Co, China owns the intellectual property. All other co-authors declares no conflict of interest.

Table 2
Pharmacokinetic parameters obtained by Phoenix WinNonlin software (mean ± S.D., n = 6).

Parameters	EDR suspension	NODS	P Value
C_{max} (ng/mL)	3290.42 ± 507.41	14510.63 ± 1507.41	<0.0001
T_{max} (mins)	15.19 ± 1.45	15.52 ± 2.42	NS
$t_{1/2}$(mins)	58.31 ± 3.52	75.98 ± 6.53	<0.001
$AUC_{0\text{-}t}$ (ng min/mL)	164185 ± 15264	943885 ± 95211	<0.0001
$AUC_{0\text{-}\infty}$ (ng min/mL)	165281 ± 16525	944726 ± 96241	<0.0001
$F_{0\text{-}t}$ (%)	100	574.89 ± 18.72	<0.0001
$F_{0\text{-}\infty}$ (%)	100	571.58 ± 25.53	<0.0001

NS: Non-significant.

Author contribution

Xin-Fu Zhou (XFZ) conceived the project, Ankit Parikh (AP), Krishna Kathawala (KK), XFZ and Sanjay Garg (SG) designed the study, AP, KK, Chun Chuan Tan (CT), XFZ performed the research, AP, KK, CT, XFZ and SG analysed the data; AP, KK, XFZ, SG wrote the paper.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpharm.2016.10.052.

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